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CHANGES IN CANINE NEUTROPHIL FUNCTION(S) FOLLOWING CELLULAR ISOLATION BY PERCOLL GRADIENT CENTRIFUGATION OR ISOTONIC LYSIS[1,2]

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ABSTRACT

Analysis of cellular effector function(s) often requires their isolation from other cellular types. Cell separatory techniques could mask, or select out, clinically important functional lesions. We examined differences in canine peripheral blood neutrophil functions, i.e. migration and H_2O_2 production, following two commonly used cell separation techniques: isotonic lysis or density gradient (Percoll) centrifugation. Separation methodology was observed to have a significant impact on both metabolic and mobility functions. In comparison to isotonic lysis, Percoll separation caused near 100% increases in random migration, near 40% decreases in chemotaxis and 70% increases in H_2O_2 production.

INTRODUCTION

Many functional and biochemical studies require large numbers of purified cells. Investigators have long sought to develop in vitro minimally manipulative methodologies allowing suitable cellular separation and recovery. Numerous new techniques have evolved some of which have included; cell sorting, electrophoresis and various affinity methods. Investigators have also attempted to make use of cellular density differences. The application of density separating techniques has resulted in three general approaches; (i) neutral density sedimentation, (ii) discontinuous-gradient, and (iii) isopycnic-gradient separations. Two of the more

frequently used separation mediums are Ficoll/Hypaque (a copolymer of sucrose and epichlorohydrin) and Percoll (colloidal silica coated with polyvinyl pyrrolidone). For many investigators Percoll has become the separation medium of choice because of it's lower viscosity (1), greater cell yields (2), noncytotoxic character (3,4), and because it is not actively phagocytized (3). Since Percoll's inception, many papers making use of its separation qualities have been published. In this report, we demonstrate differences in canine neutrophil effector functions after separation by whole-blood isotonic lysis or Percoll density gradient methods.

MATERIALS AND METHODS

Dogs: Male and female littermates (<2 years old, 10-12 kg Hra beagles) were used. Dogs were quarantined on arrival and screened for evidence of disease before being released to experiments. Animals were kenneled in an AAALAC-accredited facility in runs, and provided commercial dog chow and tap water ad libitum.

Cell Separation and Isolation: Peripheral blood samples in preservative-free heparin (10 U/ml) were drawn from the lateral saphenous vein, centrifuged (400 x g, 10 minutes) to remove plasma, diluted 2-fold in saline and divided into two aliquots. One aliquot was isotonically lysed with 0.83% NH_4Cl (4°C, 5 minutes), washed in saline and resuspended in saline supplemented with 0.2% heat-inactivated fetal bovine serum (56°C, 30 minutes). The second aliquot was layered unto a 60% (p=1.077)/70% (p=1.080) discontinuous Percoll gradient and centrifuged (700 x g, 30 minutes, room temperature). Neutrophils sedimented to the 60%/70% interface whereas mononuclear cells (i.e., monocytes, lymphocytes) remained on top of the 60% Percoll. The neutrophil layer was aspirated from the gradient and washed to remove adherent Percoll. Contaminating red blood cells in the Percoll isolate were isotonically lysed as described above, washed and resuspended in supplemented saline. On the basis of vital dye exclusion cellular viabilities were routinely >95%.

Cellular Migration: Neutrophil migration was evaluated by quantitating cells that migrated through a 10 um-thick polycarbonate membrane. A 48-well micro-chemotaxis chamber assembly (Neuro Probe Inc., Bethesda, MD) was used as previously described (5). Lower wells contained a 1:100 dilution of chemoattractant (zymosam activated plasma (6)) or buffer. Upper wells contained 50 ul of

cellular suspension which had been corrected to 2x10e6 neutrophils/ml. Chambers were incubated at 37°C in a humidified environment and gassed with 5% CO_2 and air for 1 hour. Membranes were fixed in 100% methanol for 1 minute before being stained with Dif-Quick differential stain (Fisher Scientific, Silver Spring, MD). Seven high power (100X) microscopic fields across the diameter of the well were examined and the mean cellular migration/field/hour was determined.

Quantitation of Intracellular H2O2 Production: Neutrophil H2O2 production was assayed as described by Bass et al (7). Neutrophils (1x10e6 cells/ml) were incubated with 5 uM dichlorofluorescein diacetate (DCFH-DA) for 10 minutes at 37°C. DCFH-DA traverses the cell membrane and is converted by cellular esterases into intracellularly trapped nonfluorescent DCFH. Cytosolic H2O2 endogenously present in resting cells or produced following activation, oxidizes DCFH to DCF, a fluorescent analogue. The relative fluorescence intensity of the cell is, therefore, an indirect measure of cellular H2O2 production. Cells were analyzed on a FACS analyzer (Becton Dickinson, Mountain View, CA). Green fluorescence was monitored between 515 and 545 nm after excitation with a mercury arc lamp using a 485/22 nm excitation filter. Cells were stimulated with phorbol myristate acetate (100 ng/ml, 15 minutes, 37°C) and analyzed. Neutrophils were distinguished from other cellular types on the basis of coulter volumes(s) and right angle light scatter properties.

RESULTS

The leukocyte population isolated from peripheral blood by isotonic lysis was >85% neutrophils based on differential analysis. The neutrophil population at the 60%/70% Percoll interface was >95% neutrophils. Less than 5% of the total neutrophil population remained with the mononuclear cells above the 60% Percoll layer.

No differences due to sex were apparent within the parameters examined in this report. Male: compared to separation by isotonic lysis, random migration of Percoll separated neutrophils was increased >100% (Figure 1), H2O2 production was increased by 79% (Figure 2), and chemotactic migration decreased by 37% (Figure 3). Female: Random migration of Percoll separated neutrophils increased by 95% (Figure 1), H2O2 production increased by 66% (Figure 2), and chemotactic migration (Figure 3) decreased by 43% compared with cells isolated by whole blood lysis.

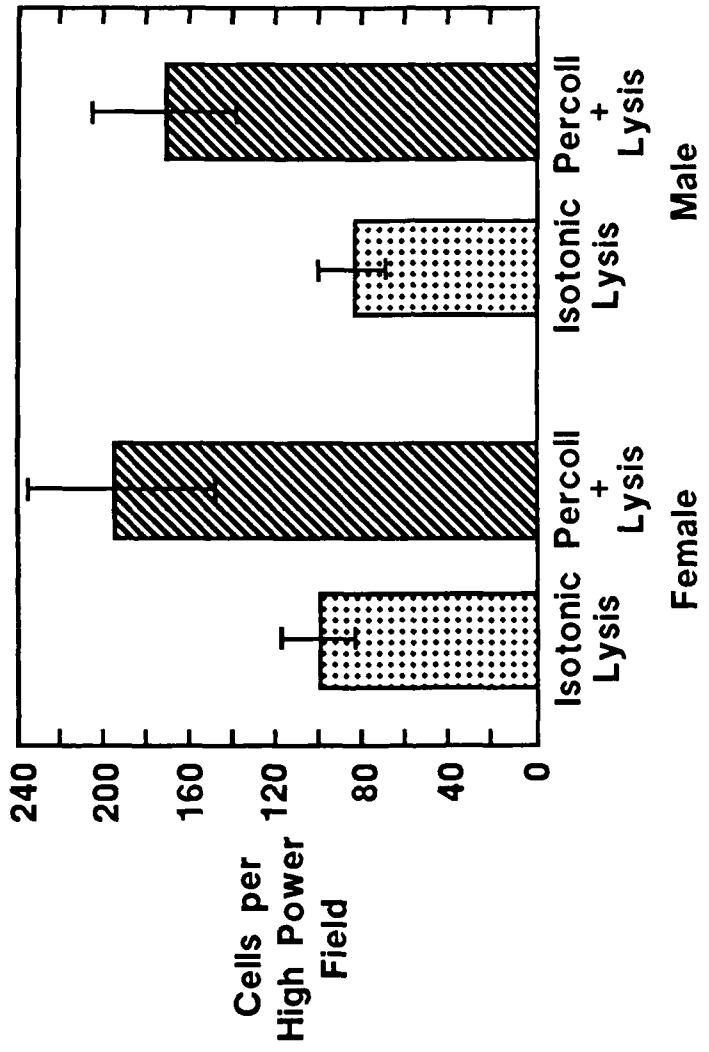

Figure 1. Random migration of canine neutrophils isolated by isotonic lysis alone or Percoll gradient separation followed by isotonic lysis. Each data point represents the mean +/- SEM number of migrating cells per high-power field from a minimum of six animals.

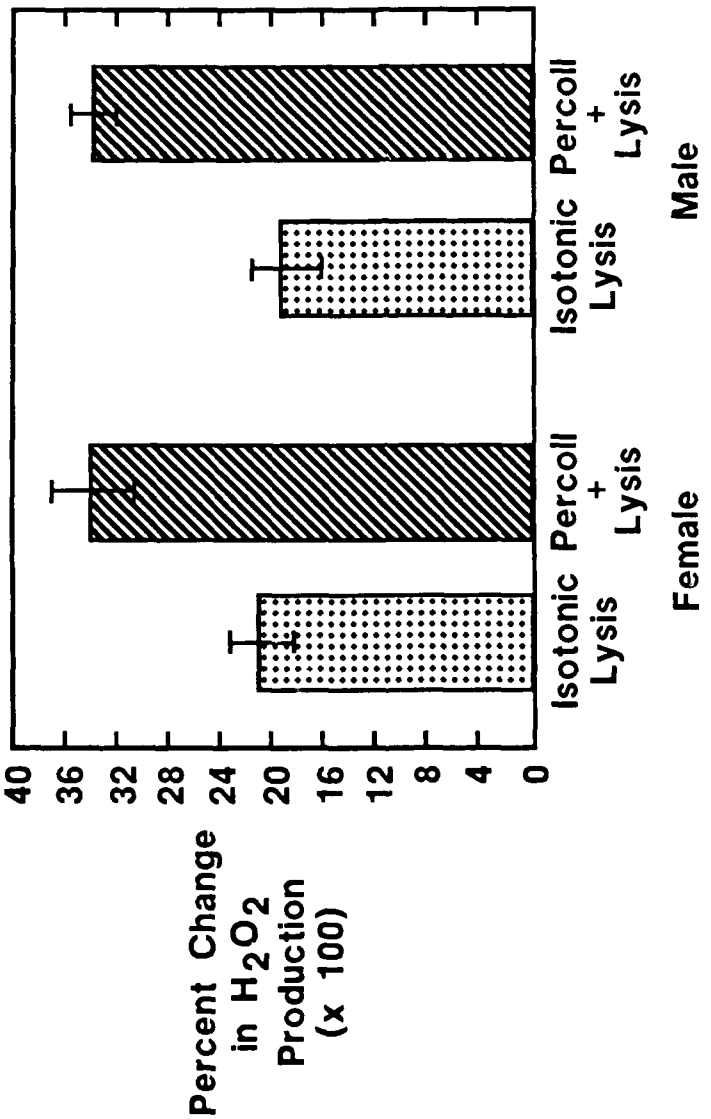

Figure 2. H202 production following isotonic lysis alone or Percoll gradient separation followed by isotonic lysis. Each data point represents the mean +/- SEM percent change in production (x100) following PMA stimulation from a minimum of six animals.

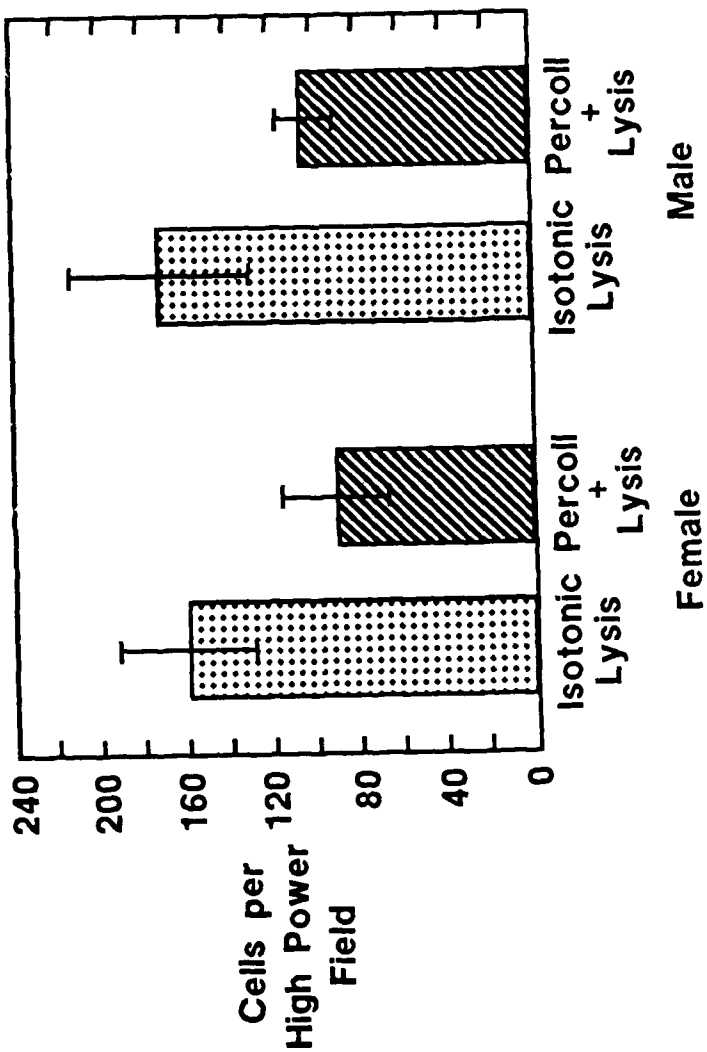

Figure 3. Chemotaxis of canine neutrophils to zymosan-activated normal dog plasma (10e-2 M) following isotonic lysis alone or Percoll gradient separation followed by isotonic lysis. Each data point represents the mean +/- SEM number of migrating cells per high-power field from a minimum of six animals.

DISCUSSION

Clinically relevant immunological dysfunction(s) may be the result of subtle cellular alterations. Unfortunately studies of specific cellular effector function(s) often require the isolation of the cell(s) of interest from other contaminating elements, cellular and otherwise. We demonstrate here that the isolation method significantly affects the metabolic and motility effector function(s) of canine neutrophils. Previous studies have reported that isolation through Ficoll-metrizoate density gradients altered membrane events so as to produce an oxidative burst (8). Based on the data presented here, separation-induced anomalies can be extended to include metabolic and motility functions as well. Percoll separation followed by isotonic lysis, in comparison to lysis alone, increased the basal motility of neutrophils evidenced by significant increases in levels of random migration. Previous reports (9,10), in conjunction with the functional differences reported here, suggest that cellular anomalies may be inherent to the manner of separation and must be considered before making judgements on apparent effector cell dysfunction(s).

FOOTNOTES

1. Supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under research work unit 00130. Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred.

2. Research was conducted according to the principles enunciated in the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Research, National Research Council.

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